

# Urinary Hypoxanthine and Xanthine Levels in Type 2 Diabetes Mellitus

## TİP 2 DİABETES MELLİTUSDA İDRAR HİPOKSANTİN VE KSANTİN DÜZEYLERİ

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### Abstract

**Objective:** Complications of the vascular tree are the leading causes of morbidity and mortality in patients with diabetes mellitus. Besides the well-recognized microvascular complications of diabetes, there is as well a growing epidemic of macrovascular complications, particularly in the type 2 diabetic population. Hyperglycemia-induced pseudo-ischemia and oxidative stress are suggested to play a role in the patho-mechanism of vascular damage. The impaired ATP metabolism observed in ischemic conditions results in increased production of the purine degradation products, hypoxanthine and xanthine. The aim of this study was to investigate the relevance of hypoxanthine and xanthine levels to microvascular complications observed in type 2 diabetic patients.

**Material and Methods:** The study included 40 patients with type 2 diabetes mellitus (29 patients were microalbuminuric and 11 were normoalbuminuric) and 30 sex-matched controls. Serum creatinine, urea, uric acid and urine creatinine, uric acid and microalbumin levels were determined. Urinary excretion of hypoxanthine and xanthine was assessed by HPLC with UV detection.

**Results:** Urinary xanthine and hypoxanthine excretions were significantly increased in the patients when compared with healthy controls ( $p < 0.05$ ). However, no significant difference was observed between the normoalbuminurics and microalbuminurics.

**Conclusion:** As a consequence of hyperglycemia-induced pseudo-hypoxia, in type 2 diabetes mellitus, ATP metabolism deteriorates and an increase in purine metabolism is observed. Urinary xanthine and hypoxanthine levels are increased in diabetic patients.

**Key Words:** Hypoxanthine, xanthine, diabetes mellitus

### Özet

**Amaç:** Diabetes mellitus damar komplikasyonları nedeni ile önemli bir morbidite ve mortalite nedenidir. Mikrovasküler komplikasyonların yanı sıra, özellikle tip 2 diabetes mellituslu (NİDDM) hastalarda, makrovasküler komplikasyonlar da ön plana geçmektedir. Hiperglisemiye bağlı psödoiskemi ve oksidatif stresin vasküler alandaki patolojilerin patogenezinde rol oynadığı düşünülmektedir. İskemik durumlarda gözlenen bozulmuş ATP metabolizması purin yıkılım ürünlerinde, hipoksantin ve ksantin, artışa neden olmaktadır.

**Gereç ve Yöntemler:** Bu çalışmada hipoksantin ve ksantin düzeylerinin tip 2 diabetes mellituslu hastalarda izlenen nefropati ile ilişkisi incelenmiştir. Bu amaç için 40 tip 2 diabetes mellituslu hasta (29 normoalbuminürlü ve 11 mikroalbuminürlü) ve 30 sağlıklı kontrol çalışmaya dahil edilmiştir. Serum kreatinin, üre, ürik asit ve idrar kreatinin, ürik asit, mikroalbumin düzeyleri belirlenmiştir. İdrar hipoksantin ve ksantin düzeyleri UV deteksiyon ile HPLC cihazında ölçülmüştür.

**Bulgular:** Tip 2 diabetes mellituslu hasta grubunda idrar ksantin ve hipoksantin atılımı sağlıklı gruba göre istatistiksel olarak anlamlı yüksek bulunmuştur. İdrar hipoksantin ve ksantin düzeyleri mikroalbuminürlü ve normoalbuminürlü hastalar arasında anlamlı fark göstermemiştir.

**Sonuç:** Tip 2 diabetes mellitusta, hiperglisemiye bağlı ortaya çıkan psödoiskemi ATP metabolizmasını bozarak purin metabolizmasında artışa yol açmaktadır. Artmış idrar ksantin ve hipoksantin düzeyleri tip 2 diabetes mellitus hastalarında yüksek seyretmektedir.

**Anahtar Kelimeler:** Hipoksantin, ksantin, diabetes mellitus

Türkiye Klinikleri J Med Sci 2004, 24:579-584

Geliş Tarihi/Received: 25.03.2003

Kabul Tarihi/Accepted: 26.08.2004

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**D**iabetes mellitus (DM) is a chronic metabolic disease characterized with hyperglycemia. Micro- and macrovascular complications of diabetes have a complex pathogenesis involving dysfunction and damage of

vascular endothelial cells which are susceptible to stimulatory factors such as increased glucose concentrations. Oxidative stress is believed to play an important role, albeit not fully recognised, in the development of vascular complications in diabetes mellitus particularly type 2 and the occurrence of oxidative stress in diabetes has been extensively documented.<sup>1,2</sup>

Under hypoxic conditions, an imbalance occurs between metabolic demand and cellular energy supply. ATP is broken down to ADP, AMP, inosine, hypoxanthine and xanthine. Xanthine oxidoreductase (XOR), which exists in the oxidase (XOD) and dehydrogenase (XDH) forms, is involved in the breakdown of hypoxanthine to xanthine and on to uric acid. Enzyme activity is induced by hypoxia and the conversion of xanthine dehydrogenase to xanthine oxidase results in increased free radicals generation. On the other hand, accumulation of hypoxanthine and xanthine which are pro-oxidant substrates for xanthine oxidase, increases the conversion of xanthine dehydrogenase to its oxidase form. In sum, increased purine catabolism ends up with increased end-products, hypoxanthine and xanthine and increased oxidative stress.

Hypoxanthine and xanthine levels in extracellular fluids have been studied extensively in many clinical conditions of oxidative stress, including perinatal asphyxia, ARDS, cerebral ischemia, tumor hyperthermia and pre-eclampsia.<sup>3-5</sup>

The aim of this study was to investigate the relevance of urinary hypoxanthine and xanthine levels in type 2 DM with respect to vascular complications.

### Material and Methods

The patient group consisted of 40 patients (26 females, 14 males, mean age  $57.64 \pm 8.6$ , mean  $\pm$  sd) who had type 2 DM. They were on diet alone (12 patients), or receiving sulphonylureas (21 patients) or biguanides (7 patients). Body weight had been stable in all subjects for at least 3 months before the study. None of the patients had a history of cerebrovascular or ischemic heart diseases,

neuropathy, 10 patients had hypertension. Twenty nine patients were microalbuminuric and eleven were normoalbuminuric. Healthy sex-matched subjects (n=30, 19 women and 11 men) served as non-diabetic controls. The mean age ( $\pm$  sd) of these subjects was  $48.1 \pm 9.3$  years. All subjects gave written, informed consent, which was approved by the Ethical Committee of Celal Bayar University, Medical Faculty.

Health status and life style patterns were assessed in detail, including purine-rich food intake and physical activity which are known to affect oxypurine excretion. Subject characteristics are presented in detail in Table 1. Serum creatinine, urea, uric acid levels were determined in venous blood samples drawn from patients upon admission after one night of fasting. Urinary creatinine, uric acid, hypoxanthine and xanthine were assessed in random urine samples upon admission and urinary hypoxanthine and xanthine were expressed as nmol/ mg creatinine. For microalbumin levels, 24-hours collected urine samples were accepted.

### Laboratory methods

Serum creatinine, urea, uric acid and urine creatinine, uric acid levels were determined by commercially available reagents on an automatic analyzer (Dax-48, Bayer Diagnostics). Urine microalbumin concentrations were determined by a

**Table 1.** Clinical data of type 2 diabetic patients and of control subjects.

	Controls (n= 30)	Patients with type 2 Diabetes mellitus (n= 40)
Age (years)	48.1 $\pm$ 5.3	57.64 $\pm$ 8.6*
Sex (F/M)	19/11	26 /14
Medication	None	
Diet		12
Sulphonylureas		21
Biguanides		7
Additional disease		
Hypertension	None	10
Life style		
Smokers	10 (33%)	15 (37%)

(Values are expressed as mean  $\pm$  sd)

\* p< 0.05.

immunoturbidimetric method on Hitachi 704 autoanalyzer.

Urine hypoxanthine and xanthine were measured by HPLC with ultraviolet detection by some modifications of a method described by Kurtz et al.<sup>6</sup> Briefly, 1 mL of urine was filtered through a 0.45 µm (pore size) Sartorius PTFE buffer, diluted 10-fold with distilled water and subjected to HPLC analysis. The column was EC 150/4.6 Nucleosil 100-5 C18 5µm (Macherey-Nagel Gmb&Co.KG, Düren, Germany). 20 µL of samples were injected and an isocratic separation at a flow rate of 1 ml/min with 20 mM potassium phosphate buffer (pH 3.0) at a column temperature of 40°C was performed. Hypoxanthine and xanthine were detected at 254 nm with an ultraviolet detector (SPD-M10A VP Shimadzu Diode Array Detector). Peak authenticity was confirmed by use of pure standarts. Quantitation was based on peak area measurements.

**Recovery:** Four urine samples were supplemented with two different standard solutions of hypoxanthine and xanthine and analyzed in triplicate. The mean recovery was 98.2% for hypoxanthine and 96.4% for xanthine.

**Precision:** To determine between-run and within-run precisions, a urine sample control containing 97 µmol of xanthine per liter was used. The coefficient of variation for within-run was determined as 5.3%, and for between-run it was

calculated as 6.8% from 40 assays performed over a 10-day period.

**Statistical Analysis**

For statistical analysis the results were subjected to parametric (student’s t) and nonparametric tests (Kruskal-Wallis, Mann-Whitney U) tests. For correlations Spearman correlation test was used. For these procedures, “Statistical Packages for Social Sciences (SPSS)” for Windows Version: 10.0 was used. Data are expressed as median: Lower quartile-upper quartile, unless stated otherwise.

**Results**

Table 1 shows the clinical data of type 2 diabetic patients and of control subjects. The mean age of the diabetic group was higher than the controls (57.64 ± 8.6 and 48.1 ± 5.3, respectively, p< 0.05). There was not a statistically significant gender difference between the two groups.

The clinical data of type 2 diabetic patients with microalbuminuria and without microalbuminuria and the control subjects are summarized in Table 2. Urinary hypoxanthine and xanthine levels were statistically different between the patients and the controls. Due to the significant difference of age between the controls and the diabetics, correlation analysis between urine analytes and age was performed, and no significance was revealed.

Microalbuminurics displayed higher values of

**Table 2.** Clinical data in type 2 diabetic patients with or without microalbuminuria and in control subjects.

	Controls (n= 30)	Normoalbuminurics (n= 29)	Microalbuminurics (n= 11)	Statistical Significance
Fasting blood glucose (mmol/L)	4.38 (4.17-5.57)	7.42 (6.12- 9.84)	10.34 (7.21-12.36)	p= 0.001 <sup>a,b,c</sup>
HbA1c (%)	5.8 (5.3-5.82)	6.9 (6.18-8.14)	7.8 (6.6-8.7)	p= 0.001 <sup>a,b,c</sup>
Microalbumin (mg/day)	8.98 (4.25-19.75)	13.8 (7.1-22.87)	102 (39.2-129)	
Serum creatinine (µmol/L)	83.98 (73.37-90.27)	92.15 (74.1-102.38)	97.9 (81.2-116.21)	p= 0.05
Serum uric acid (µmol/L)	202 (152.86-255.76)	291 (240.89-327.14)	321 (249.81-380.67)	p= 0.01 <sup>a,b</sup>
Urinary uric acid (µmol/L)	3046 (2740-4074)	3455 (2510-4585)	3307 (2557-5680)	ns
Urinary Hypoxanthine (nmol)	41.51 (33.35-47.20)	104.50 (94.07-121.61)	108.47 (95.02-128.15)	p= 0.01 <sup>a,b</sup>
Urinary Xanthine (nmol)	43.21 (37.92-46.09)	151.90 (130.8-159.7)	154.40 (142.9-162.5)	p= 0.01 <sup>a,b</sup>

Values are expressed as median (lower quartile-upper quartile)

<sup>a</sup>Normoalbuminurics vs. control,

<sup>b</sup>Microalbuminurics vs. control,

<sup>c</sup>Microalbuminurics vs. normoalbuminurics.

both hypoxanthine and xanthine when compared with normoalbuminurics, but the differences were not significant. Serum uric acid levels were higher in the patient groups, but were indifferent between the normoalbuminurics and microalbuminurics.

As expected, serum glucose and HbA1c levels were significantly higher in both microalbuminurics and normoalbuminurics than in controls ( $p= 0.001$  in each). Serum glucose levels were significantly higher in microalbuminurics than in normoalbuminuric patients ( $p= 0.001$ ). However, there were not significant differences in HbA1c values between normoalbuminuric and microalbuminuric patients.

There was a positive correlation between urinary hypoxanthine and xanthine levels in men and women ( $\rho= 0.752$  and  $\rho= 0.0.705$ , respectively,  $p= 0.0001$ ). In both gender groups, serum uric acid levels displayed positive correlations both with urinary hypoxanthine and xanthine. In men, between serum uric acid and urinary hypoxanthine levels,  $\rho$  was equal to 0.534 ( $p= 0.001$ ) and between serum uric acid and urinary xanthine levels  $\rho$  was equal to 0.434 ( $p= 0.007$ ). In women the former correlation revealed a  $\rho$  value equal to 0.516 ( $p= 0.001$ ) and the latter correlation revealed a  $\rho$  value equal to 0.498 ( $p= 0.001$ ).

In all three groups, HbA1c levels displayed positive correlations with urinary hypoxanthine levels. In the control group, the correlation coefficient was  $\rho= 0.730$  ( $p= 0.01$ ), in the normoalbuminuric group it was  $\rho= 0.814$  ( $p= 0.01$ ) and in the microalbuminuric group it was  $\rho= 0.941$  ( $p= 0.01$ ).

### Discussion

Micro- and macrovascular complications of diabetes have complex pathogenesis involving dysfunction and damage of vascular endothelial cells which are susceptible to stimulatory factors such as increased glucose concentrations. Vasodilatation and increased blood flow are characteristic early vascular responses to acute hyperglycemia and tissue hypoxia. In diabetic

patients a state of pseudoischemia is present because 2,3-bisphosphoglycerate mutase enzyme has been found to be decreased in these patients. This is a multifunctional enzyme that catalyzes both the synthesis and the degradation of 2,3-diphosphoglycerate. In humans, it occurs only in erythrocytes and plays a pivotal role in the dissociation of oxygen from hemoglobin via 2,3-diphosphoglycerate. Decreases in 2,3-diphosphoglycerate concentration within the red cell, shift the oxygen dissociation curve to the left, resulting in a high affinity of oxygen to hemoglobin reducing oxygen delivery to the tissues.<sup>7,8</sup> Bunn have shown that increased glycosylation of hemoglobin tends to shift the oxygen dissociation curve to the left, as well.<sup>9</sup> Pseudohypoxia mimics the effects of true hypoxia on vascular function and plays an important role in the pathogenesis of diabetic complications.<sup>8</sup> Hyperglycemia, also increases glucose oxidation which results in promoted oxidative stress and hypoxia.<sup>10</sup>

Under conditions of hypoxia or pseudohypoxia, electron transport system becomes inefficient and increased fatty acid oxidation results in increased NADH/NAD<sup>+</sup>. AMP is broken down to hypoxanthine and xanthine. XOR, XOD and XDH forms, is involved in the breakdown of hypoxanthine to xanthine and on to uric acid.<sup>11</sup> During a hypoxic state, conversion of xanthine dehydrogenase to xanthine oxidase is induced. Promoted degradation of AMP into hypoxanthine and xanthine results in accumulation of this end-products. Increased levels are reflected to extracellular fluids therefore, these analytes are considered as sensitive markers of hypoxia.<sup>12</sup> We have demonstrated increased levels of urinary xanthine and hypoxanthine in diabetic patients compared with healthy subjects. However, normoalbuminurics and microalbuminurics displayed similar urinary hypoxanthine and xanthine levels. Since increase in age is often associated with increased atherosclerosis, to eliminate the age factor as affecting the results, correlation analysis has been performed in the control group, and the results have revealed no

significant correlation. This result is in concordance with our previous study, in which we have investigated the age and gender dependent alterations on urinary hypoxanthine and xanthine levels in healthy subjects.<sup>13</sup> Positive correlations found in all three groups between HbA1c and urinary hypoxanthine levels indicate the relation of pseudohypoxia and chronic hyperglycemia. In our study, microalbuminurics had the strongest positive correlation between HbA1c and hypoxanthine ( $\rho=0.941$ ) supporting other studies concluding hypoxanthine and xanthine as markers of hypoxia. Even in a healthy condition, like in our control group, there was a positive correlation between above mentioned two analytes ( $\rho=0.730$ ).

Correlating with these results, serum uric acid levels were also higher in the patient groups. Although microalbuminurics displayed higher uric acid levels, the difference was not significant. This could be due to our low number of subjects included in this group. Elevated serum uric level is accepted as a feature of hyperinsulinemia/insulin resistance. It has been proposed that elevated plasma insulin concentrations may decrease urinary uric acid clearance in insulin resistant individuals. However, the relation between uric acid metabolism and type 2 diabetes remains confused because both hypouricemia and hyperuricemia are reported in different studies.<sup>14</sup> Studies suggest the existence of two different phenotypes in type 2 diabetes; the hyperuricemic insulin resistant patient with early onset or increased progression of overt diabetic nephropathy and the hypouricemic patient, less insulin resistant, with hyperfiltration and a late onset or decreased progression of overt nephropathy.<sup>15</sup>

Diabetic patients have a 2 to 5 fold increased risk of cardiovascular disease and underlying causes are multi-factorial.<sup>16</sup> Although glycemic control, blood pressure, dyslipidemia and smoking are major determinants, high uric acid levels is a common finding in subjects with cardiovascular disease. We had 8 hyperuricemic

patients with hypertension in the microalbuminuric group, indicating higher uric acid levels may be related to higher risk of cardiovascular disease in these patients.<sup>17</sup>

In summary, we have demonstrated increased levels of urinary xanthine and hypoxanthine in patients with type 2 diabetes and a strong correlation between HbA1c and urinary hypoxanthine levels. It is likely the hyperglycemia-induced pseudohypoxia causes an accumulation of this purine degradation product and leads to the conversion of XDH to XOD. XOD and its substrates, hypoxanthine and xanthine. Although urinary hypoxanthine and xanthine levels may be used as hypoxia markers, our results showed that this pathway seems to be irrelevant with the nephropathy observed in diabetes.

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